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## Toxicology in Vitro

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## *In vitro* assessment of the cytotoxic and mutagenic potential of perfluorooctanoic acid

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## ARTICLE INFO

## Article history:

Received 7 February 2008

Accepted 9 April 2008

Available online 15 April 2008

## Keywords:

Perfluorooctanoic acid

Cytotoxicity

Mutagenicity

*In vitro* bioassays

## ABSTRACT

Perfluorooctanoic acid (PFOA) is a perfluorinated compound ubiquitously detected in the environment, including wildlife and humans. Despite the available information, research on the cytotoxicity of PFOA in non-tumoral mammalian cells is relatively limited.

In this work, two *in vitro* toxicity systems were employed to provide further insight into the cytotoxic and mutagenic potential of PFOA. The cytotoxicity of the chemical towards Vero cells was assessed using biochemical and morphological parameters, while mutagenicity was evaluated according to Ames test. High doses of PFOA cause oxidative stress in Vero cells, that was closely linked to cell cycle arrest at the G1 phase and induction of apoptosis. Our results corroborate previous findings in human tumoral cells and suggest that the mode of action of this perfluorinated compound is not a peculiarity among mammalian cell types. On the other hand, the compound was not mutagenic in the Ames test, using four strains of *Salmonella typhimurium* in the presence or absence of rat S9 metabolic activation system.

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### 1. Introduction

Perfluorinated compounds (PFCs) are just one of many groups of hazardous pollutants building up in biota and water on a global scale (Prevedouros et al., 2006). These chemicals are widely used in a very broad range of industrial, commercial and consumer products, because of their non-stick and water repellent properties. The unusual physico-chemical features of PFCs, so effective in these products, also make them extremely resistant to degradation, thus raising threats to both environment and human health (EPA, 2002).

Recent biomonitoring studies reveal the prevalence of PFCs levels, particularly perfluorooctanoic acid (PFOA), in wildlife tissues from all over the world (Kannan et al., 2002) as well as in the serum of human general populations (Olsen et al., 2005). It is worth mentioning that extensive investigations do not suggest consistent association between PFOA exposure and significant human health problems (Butenhoff et al., 2004), although this compound is considered a “likely” human carcinogen (EPA, 2002).

Most toxicological studies have focused primarily on analyzing the effects of perfluorooctanoic acid in experimental animals and corresponding publications have been recently reviewed (Lau et al., 2007). Estimation of toxicity in rodents revealed that PFOA is readily absorbed but not metabolized (Kuslikis et al., 1992) and preferentially accumulated in the liver (Kudo et al., 2006). The developmental and postnatal toxicity of PFOA has also been

examined in mice (Lau et al., 2006) and rats (Hinderliter et al., 2005), although dissimilar profiles were observed due to pharmacokinetic differences between these two rodent species (Lau et al., 2006). In addition, PFOA is known to suppress immune system function in mice (Yang et al., 2002) and cause tumors in rodents (EPA, 2002).

On the other hand, cytotoxicity profiles for fish (Oakes et al., 2004) and invertebrates (Sanderson et al., 2004) indicate that, at environmental levels, PFOA elicited only moderate effects in most organisms.

Comparatively, few *in vitro* studies have been conducted to evaluate the cellular mechanisms underlying the toxicity of this perfluorinated compound in mammalian cells. Recent results obtained in rat tumor cell lines suggest that PFOA is not acutely toxic at the cellular level (Mulkiewicz et al., 2007). Even so, it has been reported that this compound perturbs the cell cycle, induces apoptosis and exerts genotoxic effects in the human hepatoma cell line HepG2 such as DNA breaks (Panaretakis et al., 2001; Shabalina et al., 1999) and micronuclei (Yao and Zhong, 2005). At present, there are no similar data available in non-tumoral mammalian cells, so renewed efforts are needed to better understand the toxic and carcinogenic effects of PFOA.

In previous studies from this laboratory it has been shown that the monkey kidney-derived cell line Vero, is a useful tool for studying the cellular effects induced by chemicals that are currently found in the environment (Fernández Freire et al., 2005; Labrador et al., 2007; Pérez Martín et al., 2008). Thus, the present work was designed to analyze the cytotoxic potential of PFOA in mammalian

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Vero cells, in order to provide further insight into the toxicological profile of this ubiquitous pollutant. The evaluation was combined with the *Salmonella typhimurium*/microsome test, predictive of integral mutagenic/carcinogenic activity.

Our results, although simplified with respect to the *in vivo* situations, indicate that PFOA was negative in the Ames test and produces moderate toxicity in normal mammalian cells.

## 2. Materials and methods

### 2.1. Cell culture and PFOA treatments

Vero cells were grown at 37 °C in 25 cm<sup>2</sup> flasks (Falcon, Becton Dickinson, USA) under a 5% CO<sub>2</sub> humidified atmosphere, using Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (all from Bio-Whittaker, Belgium). Cells were seeded at a density of 10<sup>5</sup> cells/ml in 25 cm<sup>2</sup> flasks or into 24-well plates for quantitative analysis and into 6-well plates, containing a sterile glass coverslip in each well, for morphological studies.

After removing cell culture medium and washing in phosphate buffered saline (PBS), exponentially growing cells were incubated for 24 h with new medium containing serial dilutions of PFOA. Stock solutions of 10<sup>-1</sup> M perfluorooctanoic acid (Sigma, USA), were prepared in dimethylsulphoxide (DMSO) and maintained in darkness at room temperature. The working solutions, ranging from 10 to 500 µM (4–200 µg/ml), were prepared fresh in different media according to each experimental system, and sterilized by filtration through a 0.22 µm Millipore® filter. Maximum DMSO concentration in medium was 0.5% including the control groups.

### 2.2. Cytotoxicity assessment

Two colorimetric assays were used to determine the cytotoxicity of PFOA. The tetrazolium MTT test was performed according to the method of Mosmann (1983). Briefly, following treatments with PFOA, cell cultures were incubated for 2 h with MTT (Sigma) in DMEM, at a final concentration of 0.5 mg/ml. The medium was replaced with DMSO for formazan solubilization, before reading absorbance. A second assay was the quantification of total protein content (TPC), according to the method of Bradford (1976) using Coomassie® Brilliant Blue G-250 reagent (Bio-Rad, USA) and bovine serum albumin (Sigma) as standard. Absorbances were measured at 570 and 595 nm, respectively, using a Spectrafluor microplate reader (Tecan, Austria).

### 2.3. Morphological characterization of mitochondria

For evaluation of mitochondria integrity, control and PFOA-treated cells were incubated 8 min in complete culture medium with 6.5 µg/ml rhodamine 123 (Sigma). After a thorough wash with PBS they were immediately observed under blue exciting light, as a wet preparation.

Microscopic observations were carried out using an Olympus BX-61 epifluorescence microscope (Tokyo, Japan), equipped with an HBO 100 W mercury lamp and ultraviolet (UV, 365 nm), blue (450–490 nm) and green (546 nm) excitation filters. The images were acquired with a CCD camera Olympus DP-70 and processed using the software Olympus DP controller 1.1.1.65 and Adobe Photoshop 9.0 (Adobe Systems Inc.).

### 2.4. Cell cycle analysis

To analyze cell cycle distribution, Vero cells treated for 24 h with 50, 200 and 500 µM PFOA, were collected by trypsinisation

and fixed with ice-cold 70% methanol. After gentle washing with PBS, the cells were resuspended in 1 ml of staining buffer consisting of 0.1% sodium citrate, 50 µg/ml propidium iodide and 50 µg/ml RNase A (all Sigma), and incubated in the dark for 30 min. Nuclei were then analyzed using a Coulter Epics XL-MCL flow cytometer with the Expo 32 ADC software (Beckman Coulter Inc., USA).

### 2.5. Quantification of apoptotic cells

Nuclear integrity was visualized by DNA staining with the fluorescent dye Hoechst 33258, as previously described (Fernández Freire et al., 2005). Briefly, after treatment with PFOA, the detached cells recovered in the supernatant and the adherent cells recovered by trypsinization, were pooled and centrifuged (10 min at 1200 rpm). The pellets were re suspended in 100 µl cold methanol and 10 µl (1 mg/ml) Hoechst 33258 and kept in the dark at -20 °C until used. An aliquot of each cell suspension was taken for counting, the numbers of condensed or fragmented nuclei versus normal nuclei, in at least 2000 cells for each treatment.

### 2.6. Detection of reactive oxygen species (ROS)

In order to measure the intracellular ROS level after PFOA treatments we used the 2',7'-dichlorofluorescein diacetate (DCFH-DA) method as described by Wang and Joseph (1999). In brief, cells were loaded with 100 µM DCFH-DA for 30 min and then washed with sterile PBS. DCFH-DA is non-fluorescent until hydrolyzed by intracellular esterases and readily oxidized to the highly fluorescent DCF in the presence of reactive oxygen species. Fluorescence was monitored on a Spectrafluor microplate reader with excitation and emission wavelengths of 488 and 530 nm, respectively. H<sub>2</sub>O<sub>2</sub> (500 µM, 30 min) was used as positive control. Results were normalized, for differences in cell number, by quantitation of cell protein according to Bradford assay and expressed as arbitrary fluorescence units (AFU) per percentage of protein, with respect to the untreated control.

### 2.7. Bacterial reversion assay

The test was performed using the classical plate incorporation method according to Maron and Ames (1983). Briefly, a set of tester strains of *S. typhimurium* that react preferentially with GC base pairs (TA98, TA100) or AT base pairs (TA102, TA104) were grown for 14 h in Difco bacto nutrient broth (Becton Dickinson) in a shaking incubator at 37 °C. After 48 h incubation of agar plates at 37 °C with 100 and 500 µM PFOA, in the presence or absence of rat S9 metabolic activation system (Sigma), counting of bacterial colonies was performed. The positive controls used per plate were 0.5 µg 4-nitroquinoline-N-oxide (Sigma) for TA98, 1 µg methyl methanesulphonate (Merck) for TA100 and TA102, and 50 µg methyl glyoxal (Sigma) for TA104. Sterilized 0.5% DMSO was used as the negative control. The mutation index (MI) was calculated as the number of revertants in the treatment sample (induced-spontaneous)/the number of spontaneous revertants. The concentration of PFOA was considered as mutagenic if MI was higher than 2.0.

### 2.8. Data analysis

Statistical analysis was carried out using the SPSS-11.5 software. The results were analyzed by Student's *t*-test for comparing paired samples and analysis of variance (ANOVA) with Bonferroni or Games-Howell as *post hoc* tests for multiple samples. Differences were considered statistically significant at *p* ≤ 0.05. Each data point represents the arithmetic mean ± standard deviation of at least three independent experiments. EC<sub>50</sub> values (50%

effective concentration) were obtained with the program Graph-Pad Prism 4.0 using non-linear regression.

### 3. Results

#### 3.1. Cytotoxic effects of PFOA

The effects of PFOA on the viability and proliferation of Vero cells after a 24 h treatment are presented in Fig. 1. The results from both endpoints were quite similar, showing initial toxicity at 50  $\mu$ M and reaching a plateau at 200  $\mu$ M. However, when cultures were exposed to concentrations  $\geq 200$   $\mu$ M (Student's *t*-test,  $p < 0.05$ ), significant differences were observed between the MTT assay, which measures mitochondrial metabolism, and TPC assay that reflects the total number of cells. Cell viability expressed as  $EC_{50}$  was found to be 219.6  $\mu$ M for MTT assay, whereas no  $EC_{50}$  values were obtained in Bradford assay with the PFOA concentrations tested in the present study.

The interference of the compound with mitochondrial integrity was verified by the morphological changes visualized under the fluorescence microscope (Fig. 2). A minor dose-dependent mitochondrial fission, characterized by the conversion of tubular fused mitochondria into isolated small organelles, was observed in cells treated with PFOA concentrations  $\geq 50$   $\mu$ M.

To evaluate whether the reduced cell density after PFOA exposure was associated with an antiproliferative effect, we conducted a flow cytometric analysis of DNA content in cells treated for 24 h with three representative concentrations of the compound. As shown in Fig. 3, moderate concentrations slightly, but not significantly, altered cell cycle progression when compared with untreated controls. However, the treatment of Vero cells with

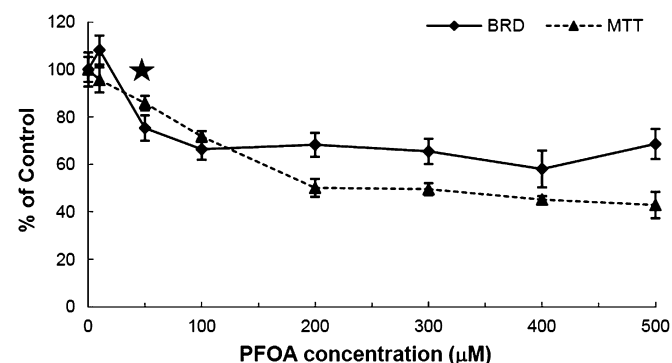


Fig. 1. Dose-response curves obtained in the two cytotoxicity assays, after treatment of Vero cells with increasing PFOA concentrations. The values represent mean  $\pm$  SD from three independent determinations. Asterisks indicate statistically different values in treated cells compared to control cells.

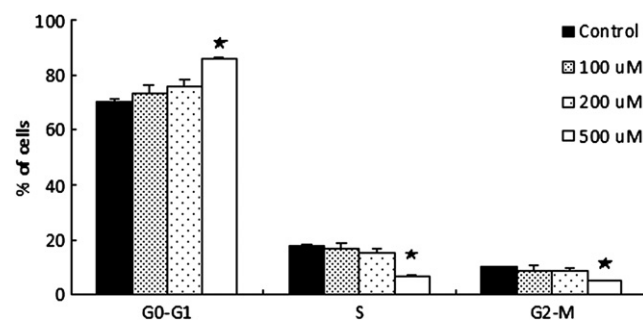


Fig. 3. Cell cycle profiles of Vero cells exposed for 24 h to increasing concentrations of PFOA. The values represent mean  $\pm$  SD of three separate experiments. Asterisks indicate statistically different values in treated cells compared to control cells.

500  $\mu$ M PFOA caused significant G0/G1 cell cycle arrest, with a concomitant decrease in the proportion of cells in the S and G2/M phases.

In addition, a dose-dependent detachment of cells from culture substrate was observed after exposure to PFOA. As apoptotic cells rapidly lose adhesion properties in many experimental *in vitro* systems, fluorescent microscopy was used to realize whether floating cells exhibited signs of cell death. Vero cells, exposed to concentrations  $\geq 50$   $\mu$ M PFOA, showed characteristic features of apoptosis including condensed and fragmented nuclei (Fig. 4a). The proportion of apoptotic cells was significant only after exposure to 500  $\mu$ M PFOA (Fig. 4b), and typically around 1–2% in the control cell population.

#### 3.2. Intracellular ROS production

The capacity of PFOA to cause oxidative stress in Vero cells was evaluated by a converting reaction of DCFH-DA to DCF, which mainly derives from the intracellular production of hydrogen peroxide. Cell cultures treated for 24 h with the test compound showed an enhanced DCF fluorescence over the control level, that was significant only at the highest concentration tested (Fig. 5). These results indicate that PFOA stimulated the generation of reactive oxygen species in a dose-dependent manner.

#### 3.3. Ames mutagenicity test

The number of revertants induced by PFOA, with or without metabolic activation (S9), are summarized in Table 1. According to these results, the mutation index was calculated and PFOA did not display mutagenic activity on the four *S. typhimurium* strains used in the present study, in either of the tested concentrations. The positive controls yielded appropriate revertant colony numbers, confirming proper strains function.

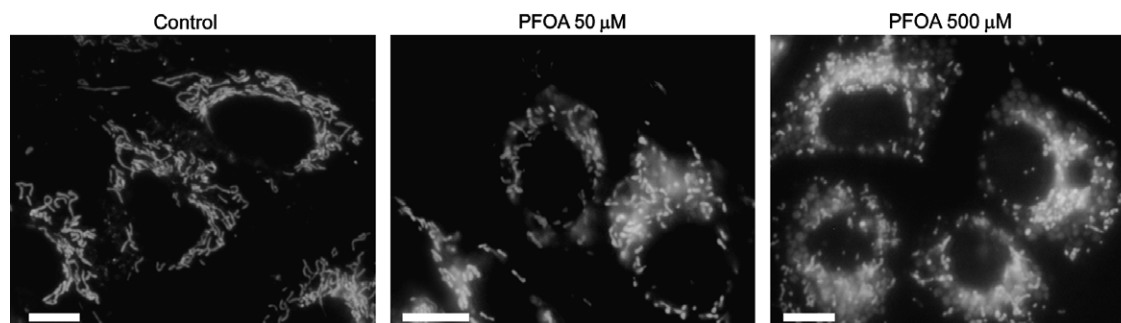
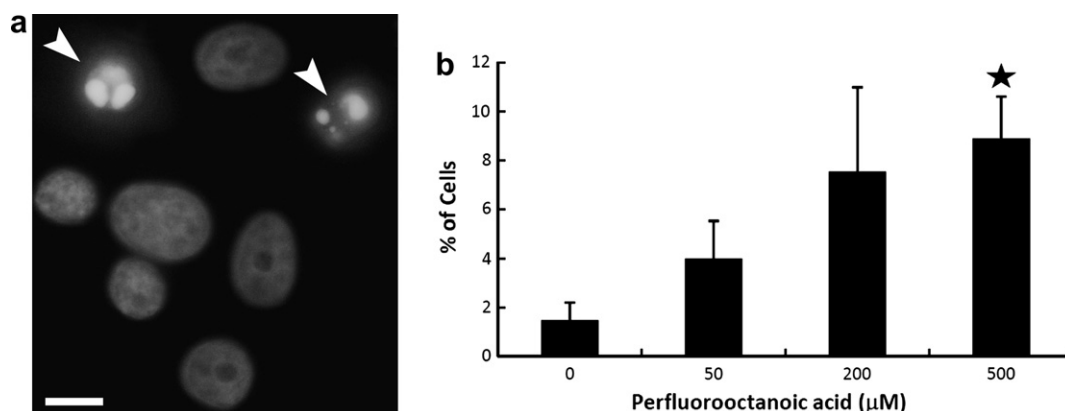
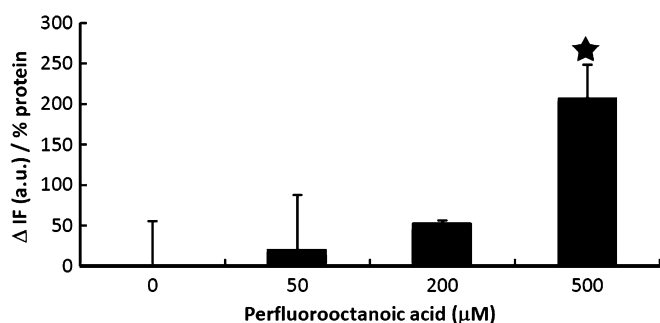


Fig. 2. Representative images of Vero cells labelled with Rh-123, showing the effect of PFOA on mitochondrial morphology. The filamentous network observed in control cells was fragmented after incubation with the compound for 24 h. Bars, 10  $\mu$ m.



**Fig. 4.** Effect of PFOA on nuclear morphology of Vero cells. (a) Fluorescence micrograph of cells, stained with Hoechst 33258 after exposure to 500 μM PFOA, showing apoptotic nuclei (arrowheads). Bar, 5 μm. (b) Quantitative data represent the rate of apoptotic cells in cultures treated during 24 h with different PFOA doses. The values are expressed as mean  $\pm$  SD of three separate experiments. Asterisks indicate statistically different values in treated cells compared to control cells.



**Fig. 5.** ROS generation estimated by increase of DCF fluorescence in Vero cells, after 24 h exposure to various concentrations of PFOA. The values represent mean  $\pm$  SD of three independent experiments. Asterisks indicate statistically different values in treated cells compared to control cells.

#### 4. Discussion

Over the last two decades, there has been increasing scientific concern regarding the occurrence and dispersion of perfluorinated chemicals in the environment. However, despite the epidemiological studies, evidence relating to the toxicity of these compounds to humans is far less conclusive. It is widely accepted that *in vitro* systems are useful for specific mechanistic studies and should be considered as a starting point in the assessment of *in vivo* toxicity. Moreover, PFOA is metabolically inert in mammals and therefore a particularly suitable compound for *in vitro* toxicity testing. In this study, a mammalian cell line and a bacterial bioassay were employed to evaluate the cytotoxicity and mutagenic potential of perfluorooctanoic acid.

By comparing cell viability and proliferation data, we observed concordance between both endpoints when cells were treated with low PFOA doses. However, concentrations around 200 μM reduced by 50% the ability of Vero cells to metabolize MTT, when about 70% of cell confluence was estimated using Bradford protein assay. These first results corroborate the well-documented inhibitory activity of PFCs on mitochondria (Starkov and Wallace, 2002), and were further supported by our subsequent studies. Following PFOA treatments, Vero cells displayed fragmentation of mitochondrial reticulum, a morphological change consistent with defective bioenergetic metabolism (Lyamzaev et al., 2004; Zorov et al., 2005). There was also evidence of intracellular H<sub>2</sub>O<sub>2</sub> generation, expressed as a dose-dependent rise in DCF fluorescence, although the statistical analysis revealed significant results only at the higher PFOA concentration tested (500 μM).

Reactive oxygen species (ROS) originating from mitochondria may be derived through respiratory chain and normally exists in all aerobic cells in balance with biochemical antioxidants. Alternatively, dysfunctional mitochondria will produce increased levels of ROS, creating a significant threat for the major classes of biomolecules primarily lipids, proteins and DNA (Zorov et al., 2005). Despite the fact that reactive oxygen species have been identified as major contributors to damage in biological systems, ROS are now recognized as potent second messengers to trigger signal transduction pathways that regulate specific processes in virtually all cells (Genestra, 2007). Current information indicates that ROS, in particular hydrogen peroxide promote either proliferation or cycle arrest and subsequent cell death, depending on the intracellular levels and/or the cell lines tested (Boonstra and Post, 2004).

The results presented in this study reveal that significant increases in the levels of intracellular H<sub>2</sub>O<sub>2</sub> were closely linked to cell cycle arrest at the G1 phase and induction of apoptosis. Accord-

**Table 1**

Number of revertants induced by PFOA with or without metabolic activation (S9)

Treatment	Revertant per plate							
	TA98		TA100		TA102		TA104	
	–S9	+S9	–S9	+S9	–S9	+S9	–S9	+S9
–	26.7 $\pm$ 7.6	70.3 $\pm$ 4.0	51.7 $\pm$ 14.0	78.0 $\pm$ 15.1	318.0 $\pm$ 26.2	382.3 $\pm$ 18.0	333.7 $\pm$ 17.0	357.7 $\pm$ 27.6
Solvent control (100 μL/plate)	23.0 $\pm$ 3.5	74.3 $\pm$ 5.9	60.3 $\pm$ 3.1	72.0 $\pm$ 7.0	290.0 $\pm$ 32.0	374.3 $\pm$ 34.0	311.7 $\pm$ 23.2	330.7 $\pm$ 14.6
Positive control <sup>a</sup>	227.3 $\pm$ 17.2	444.0 $\pm$ 27.8	500.0 $\pm$ 22.6	584.7 $\pm$ 37.5	3505.7 $\pm$ 106.2	2640.3 $\pm$ 203.8	3103.3 $\pm$ 134.2	1343.0 $\pm$ 33.8
PFOA 100 μM	21.0 $\pm$ 1.0	63.0 $\pm$ 4.4	38.3 $\pm$ 2.5	89.0 $\pm$ 5.3	321.3 $\pm$ 29.0	348.7 $\pm$ 18.0	330.0 $\pm$ 22.6	458.7 $\pm$ 65.0
PFOA 500 μM	16.0 $\pm$ 1.0	64.0 $\pm$ 6.9	47.0 $\pm$ 2.6	78.3 $\pm$ 7.0	299.0 $\pm$ 19.7	363.7 $\pm$ 23.8	337.7 $\pm$ 14.0	374.3 $\pm$ 51.5

Results are shown as the mean  $\pm$  SD of three independent experiments.

<sup>a</sup> 4-Nitroquinoline-N-oxide (TA98). Methylmethane-sulfonate (TA100 y TA102). Methyl-glyoxal (TA104).



ingly, we could hypothesize that high concentrations of PFOA cause severe oxidative stress in Vero cells. In support of this assumption, it must be stated that other authors have previously reported nearly identical findings, using human hepatoma HepG2 cells (Panaretakis et al., 2001; Shabalina et al., 1999). Taking into account that PFOA triggers a common cytotoxic response in tumoral and non-tumoral cells, it is tempting to assume that the mode of action of this perfluorinated compound is not a peculiarity among mammalian cell types. However, further studies are needed to confirm this initial proposal, as well as to verify if the PFOA effects observed in Vero cells are consistently found in other non-tumoral cell lines of mammalian origin.

Another important question which remains to be resolved is whether PFOA, in the general environments, represents a carcinogenic risk to humans. Although some medical surveillance studies have failed to detect any significant association between PFOA exposure and cancer incidence, current data are limited and still under debate (EPA, 2002). Rodent bioassays have shown that this compound induces a variety of tumor types (liver, testes and pancreas), that are in part attributed to peroxisome proliferation (Nilsson et al., 1991). Nevertheless, the relevance to humans of this precise effect of PFOA, depending on the PPAR $\alpha$  (peroxisome proliferator-activated receptor alpha), has often been questioned (Klaunig et al., 2003).

Carcinogens are known to operate through a wide range of mechanisms within the cells and consequently, several experimental methods can be employed in human risk assessment (Williams, 2001). Bacterial mutagenesis systems are useful tools in the first step of hazard identification, since a mutagenic event is accepted as a critical point in the initial phase of carcinogenesis (Davidson et al., 2003). Based on this consideration, the second part of our study was aimed to evaluate the mutagenic activity of PFOA using the *Salmonella*/microsome assay, primarily developed to predict human carcinogenicity of environmental pollutants (Maron and Ames, 1983). Previous studies have demonstrated that PFOA was not mutagenic in standard strains of *S. typhimurium* (Griffith and Long, 1980). Therefore, we decided to use alternative tester strains to gain an insight into possible mechanisms of PFOA-induced mutagenesis. It is worth emphasizing that TA102 and TA104 strains were included in our analysis, since they are capable to detect oxidant mutagens and allow the screening of carcinogens that were negative in standard tester strains (Levin et al., 1982).

Consistent with the preceding conclusions, Ames test revealed negative results in all the *Salmonella* strains used, which corroborates that PFOA is probably not a mutagen. However, this does not necessarily mean that this compound is not a carcinogen, since evidence suggests that cancers result from genetic alterations as well as from tumor promotion (Williams, 2001). Indeed, peroxisome proliferators, including PFOA, are in general tumor promoters rather than initiators (Lake, 1995). Moreover, in human HepG2 cells this compound exerts genotoxic effects as a consequence of oxidative stress and not from direct DNA damage (Yao and Zhong, 2005). Therefore, until definite evidence relating to PFOA carcinogenicity is presented, one might assume that human tumors, if produced, could arise through epigenetic mechanisms.

Taking our results together, we conclude that PFOA, at concentrations several orders of magnitude above the average level detected in human serum, shows no evidence of mutagenic activity and exerts only moderate cytotoxic effects in non-tumoral mammalian cells. However, given the stability and extended retention time of the compound in humans, further research is required, since long-term health consequences of a constant and unwitting exposure to PFOA have not yet been identified. Likewise, in view of the fact that humans are simultaneously exposed to a large number of environmental pollutants, the possible synergistic or antagonistic effects between PFOA and other chemical compounds in the body must be considered in future toxicological studies.

## Acknowledgements

This work was supported by the Spanish Ministry of Education and Science (CTM2005-02135) and the Community of Madrid (CCG06-UAM/AMB-0186). The authors thank Sylvia Gutierrez Erlandsson from the Citometry and confocal microscopy service at the Centro Nacional de Biotecnología for technical assistance.

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